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Stereoselective Metabolism of Dibenz(*a,h*)anthracene to *trans*-Dihydrodiols and Their Activation to Bacterial Mutagens

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Dibenz(*a,h*)anthracene (DBA), a carcinogenic, polycyclic aromatic hydrocarbon ubiquitous in the environment, is metabolized by the hepatic microsomal fraction of immature Sprague-Dawley rats pretreated with Aroclor 1254 to 27 ethyl acetate-extractable metabolites. More than half of these metabolites (51%) consisted of *trans*-1,2-; -3,4-; and -5,6-dihydrodiols including their identified secondary metabolites. The three *trans*-dihydrodiols (4.9, 15.8, and 0.6% of total metabolic conversion) were highly enriched in their *R,R* enantiomers (85, 71, and 98%) as determined by high performance liquid chromatography on suitable chiral stationary phases. This is explained on the basis of the stereoselective epoxidation of DBA by cytochrome P-450*c* (induced by Aroclor 1254) followed by regioselective hydration catalyzed by microsomal epoxide hydrolase. Determination of the bacterial mutagenicity by measuring the reversion rate of histidine-dependent *Salmonella typhimurium* TA100 to histidine prototrophy revealed marked differences in the mutagenicity of the enantiomers of the *trans*-dihydrodiols of DBA when activated by the same metabolizing system as used in the metabolism studies. In the case of *trans*-1,2- and -5,6-dihydrodiol, the *S,S* enantiomers were converted to more mutagenic metabolites than their corresponding optical antipodes, whereas in the case of *trans*-3,4-dihydrodiol it was the *R,R* enantiomer that produced the stronger mutagens. Therefore, both regio- and stereoselectivity of the metabolizing enzymes attribute to the dominant role of *trans*-3,4-dihydrodiol in the mutagenicity of DBA.

Introduction

Dibenz(*a,h*)anthracene (DBA) (Fig. 1), a polycyclic aromatic hydrocarbon (PAH) of considerable tumorigenicity (*1*), occurs ubiquitously in the environment as a product of incomplete combustion of organic matter. DBA has been identified in mainstream cigarette smoke (*2*), gasoline engine exhaust tar (*3*), coal-heating exhaust (*4*), urban air (*5*), and even in charcoal-broiled meat (*6*), edible oils (*7*), and vegetables (*8*).

PAHs are generally biologically inert and exhibit their genotoxic properties only after metabolic conversion to chemically reactive intermediates. DBA is enzymatically transformed in a regio- and stereoselective fashion to a great number of metabolites (*9–12*), whose contribution to the mutagenic and/or tumorigenic potential of DBA have not yet been fully elucidated.

DBA is enzymatically attacked by the cytochrome P-450-dependent monooxygenase system at three distinct molecular regions, i.e., the bay, M, and K region, yielding the arene oxides at the 1,2; 3,4; and 5,6 position (Fig. 1). The arene oxides may spontaneously aromatize to phenols or undergo epoxide hydrolase-catalyzed hydration to *trans*-dihydrodiols (*10*). Both enzymes involved in the generation of the chiral *trans*-dihydrodiols can act in a regio- and/or stereoselective manner thus

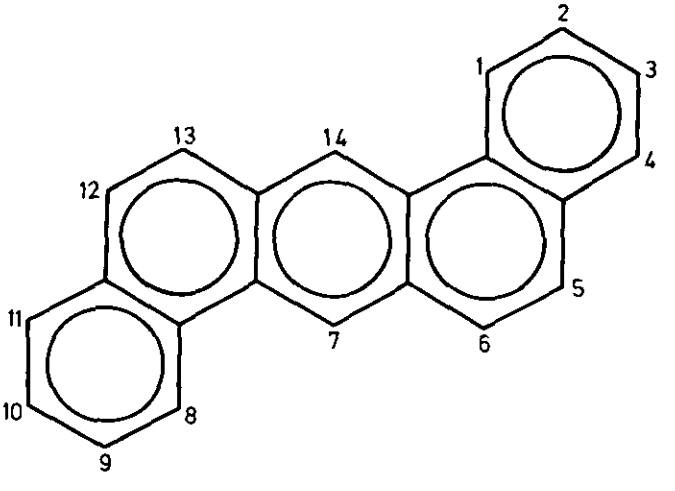

FIGURE 1. Structural formula and numbering of DBA.

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leading to enantiomeric enriched *trans*-dihydrodiols that can be further metabolized to mutagenic and/or tumorigenic secondary metabolites.

The goal of the present study was to determine the absolute configuration of the principal enantiomer of the *trans*-1,2-; -3,4-; and -5,6-dihydrodiols of DBA, metabolically formed by the hepatic microsomal fraction of Sprague-Dawley rats pretreated with the polychlorinated biphenyl mixture Aroclor 1254, and to estimate the influence of the enantiomeric enrichment on the mutagenicity of the *trans*-dihydrodiols and hence of DBA by determining the bacterial mutagenicity of the pure synthetic enantiomers under the metabolic conditions mentioned above.

Regio- and Stereoselective Metabolic Conversion of DBA to *trans*-Dihydrodiols

Incubation of [5,12-^{14}C]DPA (100 μM) with the hepatic microsomal fraction (1 mg protein/mL incubation volume) of Aroclor 1254-pretreated immature Sprague-Dawley rats in the presence of an NADPH-regenerating system at 37°C for 20 min yielded 27 metabolites after extractive work-up with ethyl acetate followed by HPLC separation (*10*). Twelve metabolites were formed in amounts sufficient to allow radiometric quantification. These metabolites are grouped in Table 1 according to their origin from the primarily formed arene oxides at the 1,2-; 3,4-; and 5,6-position of DBA (*10*). The M region (3,4-position) in DBA, accounting for almost one-third of the total metabolism, is by far the most favored site of attack of Aroclor 1254-inducible monooxygenases, while the metabolism at the bay region (1,2-position) and the K region (5,6-position) amounts to just 15 and 10%, respectively.

The *trans*-3,4-dihydrodiol is found not only as the main representative of the dihydrodiols but also, at 15.8%, as the principal microsomal metabolite of DBA. The *trans*-1,2- and -5,6-dihydrodiols can only be detected at 4.9% and 0.6%, respectively, of the total metabolic conversion. However, in evaluating the quantity of a metabolite it has to be taken into account that the amount which is detected represents the result of formation and further enzymatic conversion of that metabolite. Considering this fact, the total amount of *trans*-1,2- and -5,6-dihydrodiol originally formed can be calculated from Table 1 to be 8.6 and 8.1%, respectively. This calculation, however, only considers the identified secondary metabolites of the *trans*-dihydrodiols and still neglects the unknown water-soluble and protein-bound metabolites.

To evaluate the enantiomeric composition of the metabolically formed *trans*-dihydrodiols of DBA, two tasks had to be accomplished; determination of the absolute configuration of the enantiomeric *trans*-dihydrodiols and efficient chromatographic separation of the enantiomers. The absolute configuration was assigned to the enantiomers of the *trans*-dihydrodiols by applying two independent spectroscopic methods (*15*) showing that *R,R* absolute configuration is associated with negative optical rotation in the case of the 1,2- and 3,4-isomer and associated with positive optical rotation in the case of the 5,6-isomer.

Table 1. Metabolic conversion of DBA by liver microsomes of Sprague-Dawley rats pretreated with Aroclor 1254.[a]

Metabolite	Metabolite formation, nmole/nmole P-450 × 20 min[b]
1-Hydroxy-DBA	0.16 ± 0.01 (1.0)[c]
2-Hydroxy-DBA	0.83 ± 0.04 (5.3)
trans-1,2-Dihydroxy-1,2-dihydro-DBA	0.77 ± 0.05 (4.9)
r-1,*t*-2,*t*-3,*c*-4-Tetrahydroxy-1,2,3,4-tetrahydro-DBA	0.21 ± 0.01 (1.3)
r-1,*t*-2,*c*-3,*t*-4-Tetrahydroxy-1,2,3,4-tetrahydro-DBA	0.37 ± 0.01 (2.4)
4-Hydroxy-DBA	1.73 ± 0.28 (11.0)
4,11-Dihydroxy-DBA	0.42 ± 0.02 (2.7)
trans-3,4-Dihydroxy-3,4-dihydro-DBA	2.48 ± 0.17 (15.8)
5,6-Epoxy-5,6-dihydro-DBA	0.37 ± 0.01 (2.4)
trans-5,6-Dihydroxy-5,6-dihydro-DBA	0.09 ± 0.01 (0.6)
r-5,*t*-6,11-Trihydroxy-5,6-dihydro-DBA	0.68 ± 0.03 (4.3)
trans,trans-5,6;10,11-Tetrahydroxy-5,6,10,11-tetrahydro-DBA	0.51 ± 0.02 (3.2)
Water-soluble metabolites[d]	4.22 ± 0.41 (26.8)
Protein-bound metabolites[e]	1.50 ± 0.03 (9.5)
Total metabolic conversion	15.73 ± 0.50 (100.0)

[a] [5,12-^{14}C]DBA (100 μM) was incubated as described (*10*) with liver microsomes from immature male Sprague-Dawley rats pretreated IP with Aroclor 1254 (300 mg/kg body weight) 6 days before sacrifice.
[b] Values are means ± SD; $n = 4$.
[c] Values in parentheses refer to percentage of total metabolic conversion.
[d] Calculated from the radioactivity remaining in the aqueous phase of the incubation mixture after extraction with ethyl acetate and removal of protein.
[e] Calculated from the radioactivity bound to microsomal protein after extensive washing with methanol.

Direct chromatographic separation of the enantiomers of the *trans*-dihydrodiols was achieved using HPLC on stationary phases containing chiral π-acceptor ligands that were prepared according to described procedures (*13*). In all cases the *trans*-dihydrodiols with *R,R* absolute configuration were eluted from these chiral columns first (*10*) (Fig. 2).

Determination of the enantiomeric composition of the enzymatically formed *trans*-dihydrodiols by chiral phase chromatography according to Figure 2 revealed that the conversion of DBA to these metabolites by liver microsomes of immature Sprague-Dawley rats pretreated with Aroclor 1254 proceeds with remarkable stereoselectivity (Table 2) leading to *trans*-dihydrodiols highly enriched in the *R,R* enantiomers. This has also been observed with other PAH species (*16,18*).

trans-Dihydrodiols of PAH are formed by the sequential attack of cytochrome P-450-dependent monooxygenase and microsomal epoxide hydrolase, both enzymes acting in a regio- and/or stereoselective manner.

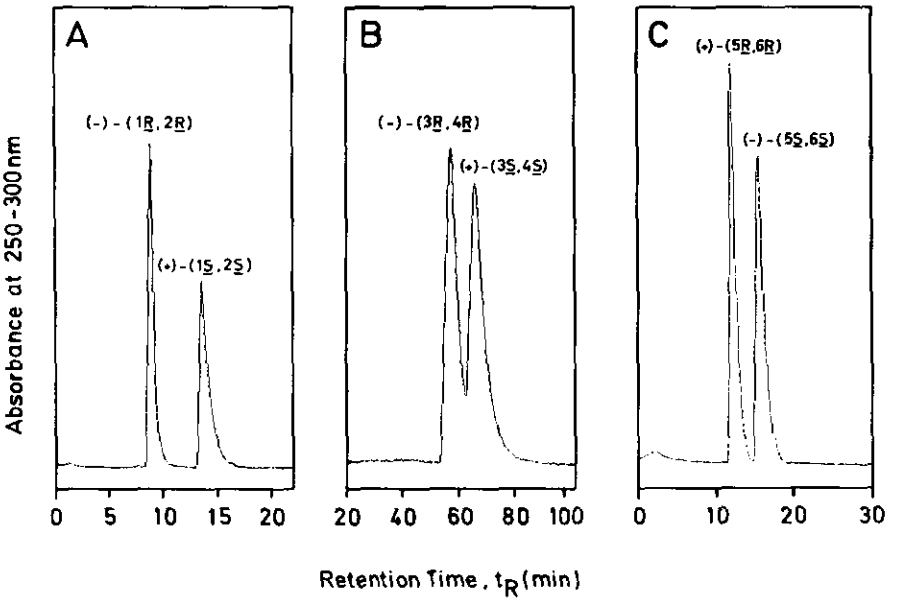

FIGURE 2. Separation of racemic *trans*-dihydrodiols of DBA into the enantiomers by HPLC on chiral stationary phases, (*A*) (±)-*trans*-1,2-dihydroxy-1,2-dihydrodibenz(*a*,*h*)anthracene; (*B*) (±)-*trans*-3,4-dihydroxy-3,4-dihydrodibenz(*a*,*h*)anthracene; (*C*) (±)-*trans*-5,6-dihydroxy-5,6-dihydrodibenz(*a*,*h*)anthracene. Stationary phases: (*A*) (–)-(*R*)-2-(2,4,5,7-tetranitro-9-fluorenylideneaminooxy)propionic acid; (*B*) (–)-(*R*)-2-(2,5,8,10-tetranitro-7-benzo(*c*)fluorenylideneaminooxy)propionic acid; (*C*) (–)-(*R*)-2-(2,4,5,7-tetranitro-9-fluorenylideneaminooxy)butyric acid. All chiral ligands were covalently linked over γ-amino-propyl spacers to silica gel (LiChrosorb Si 100, 5 μm; column dimensions: 4 × 250 mm) as described earlier (*13*). Mobile phase consisted of dichloromethane/methanol (*A*) 65:35, v/v; (*B*) 80:20, v/v; (*C*) 40:60, v/v; with a flow rate of 2.0 mL/min.

Table 2. Enantiomeric composition of *trans*-dihydrodiols of DBA formed by liver microsomes of Sprague-Dawley rats pretreated with Aroclor 1254.

Metabolite[a]	%(*R*,*R*)	%(*S*,*S*)
trans-1,2-Dihydroxy-1,2-dihydro-DBA	85 ± 2[b]	15 ± 2
trans-3,4-Dihydroxy-3,4-dihydro-DBA	71 ± 2	29 ± 2
trans-5,6-Dihydroxy-5,6-dihydro-DBA	98 ± 1	2 ± 1

[a] The *trans*-dihydrodiols were chromatographically isolated (*10*) from incubations described in Table 1, separated into the enantiomers as shown in Figure 2, and radiometrically quantitated.
[b] Values are means ± SD; $n = 4$.

The high enantiomeric purity of the *trans*-5,6-dihydrodiol of DBA is based on the almost exclusive formation of the (5*S*,6*R*)-oxide (*12*) predicted by the model of the catalytic binding site of cytochrome P-450c [P450 IA1 according to the recently recommended nomenclature (*17*)] (*18*,*19*). This isozyme of the monooxygenase system is induced in rats by Aroclor 1254 (*20*).

In contrast to the K-region arene oxide of benzo(*a*)pyrene (*21*), the (5*S*,6*R*)-oxide of DBA is enzymatically hydrated by microsomal epoxide hydrolase in a regioselective fashion by attack of water at C_5 yielding to almost pure (5*R*,6*R*)-dihydrodiol (*12*).

Due to the unfavorable binding mode of the DBA molecule (*10*,*18*) to the catalytic binding site of P-450c (*19*) for epoxidation at the 1,2 and 3,4 positions, in contrast to the 5,6-position, a lower enantiomeric purity of the 1,2- and 3,4-oxides can be expected. Assuming that these epoxides, like all non-K-region arene oxides investigated so far, are regioselectively hydrated by epoxide hydrolase-catalyzed water attack at the allylic carbon (*16*,*18*), a somewhat less marked enrichment in the metabolically formed (1*R*,2*R*)- and (3*R*,4*R*)-dihydrodiols should be expected. This has indeed been shown to be the case (Table 2).

Bacterial Mutagenicity of the Pure Enantiomers of *trans*-Dihydrodiols of DBA

The bacterial mutagenicity of the pure enantiomers of *trans*-dihydrodiols of DBA obtained by preparative HPLC of the synthetic racemic compounds (*22*,*23*) on chiral stationary phases as depicted in Figure 2 was determined by measuring the reversion rate of *Salmonella typhimurium* TA100 from histidine dependence to histidine prototrophy (*14*,*24*). The enantiomeric *trans*-dihydrodiols were metabolically activated using the same hepatic microsomal system employed to determine their regio- (Table 1) and stereoselective (Table 2) formation from DBA, and they exhibited the mutagenic effects presented in Figure 3. All three *trans*-dihydrodiols showed marked differences in the mutagenicity of the *R*,*R* and *S*,*S* enantiomers, while the corresponding values for the racemates lay between (data not shown) and were in excellent agreement with results reported earlier (*25*).

The (1*R*,2*R*)-dihydrodiol was only weakly activated to bacterial mutagens, whereas its optical antipode exhibited a moderate mutagenic effect of 500 his^+ rev-

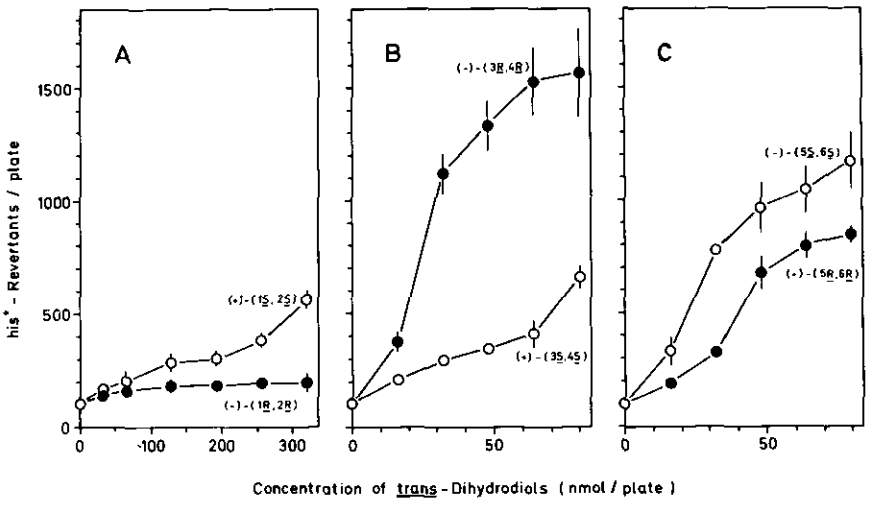

FIGURE 3. Bacterial mutagenicity of the enantiomeric *trans*-dihydrodiols of DBA determined by reversion of *Salmonella typhimurium* TA100 from histidine auxotrophy to histidine prototrophy. (*A*) (+)- and (−)-*trans*-1,2-dihydroxy-1,2-dihydrodibenz(*a,h*)anthracene; (*B*) (+)- and (−)-*trans*-3,4-dihydroxy-3,4-dihydrodibenz(*a,h*)anthracene; (*C*) (+)- and (−)-*trans*-5,6-dihydroxy-5,6-dihydrodibenz(*a,h*)anthracene. The compounds, dissolved in 30 μL *N,N*-dimethylformamide, were tested essentially as described (*14*) in the presence of liver microsomal protein equivalent to 1 nmole cytochrome P-450 per plate from immature male Sprague-Dawley rats pretreated IP with Aroclor 1254 (300 mg/kg body weight) 6 days before sacrifice.

ertants at a concentration of 300 nmole per plate. Of the enantiomers of the M region, *trans*-dihydrodiol the 3*S*,4*S* isomer was considerably activated to mutagens while the 3*R*,4*R* isomer was much more mutagenic by a factor of almost 4. This is in contrast to results obtained with the M region *trans*-7,8-dihydrodiol of benzo(*a*)pyrene where the (−)-7*R*,8*R* enantiomer was activated to weaker mutagens for *S. typhimurium* TA100 than the (+)-7*S*,8*S* enantiomer (*26*).

The difference in the mutagenicity of the (5*R*,6*R*)- and (5*S*,6*S*)-dihydrodiols was somewhat less pronounced, the *S,S* enantiomer being more strongly activated to bacterial mutagens than its optical antipode by a factor of 1.5. The strong bacterial mutagenicity of the *trans*-5,6-dihydrodiol of DBA, unusual for a K-region dihydrodiol of PAH, has already been observed when liver microsomes of immature Long-Evans rats after Aroclor 1254-pretreatment (*25*) or of C3H mice pretreated with phenobarbital were used as metabolizing system (*14*). However, mutagenicity was not observed when adult Sprague-Dawley rats were employed (*11*). It can thus be concluded that the metabolic activation of the K-region dihydrodiol of DBA to bacterial mutagens by the hepatic microsomal fraction does not depend on different rat strains as previously assumed (*11*), but is highly age dependent, at least in the Sprague-Dawley rat.

In the case of the *trans*-3,4- and -5,6-dihydrodiol of DBA it is tempting to speculate on the structure of their ultimate mutagenic metabolite(s). The *trans*-3,4-dihydrodiol, like other M-region dihydrodiols of PAH, could undergo monooxygenase-catalyzed epoxidation at the olefinic double bond, leading to a highly reactive vicinal dihydrodiol epoxide capable of covalently binding to DNA. Due to their reactivity, dihydrodiol epoxides are usually not detected as such in microsomal incubations but are detected as their hydrolysis products, the tetrahydrotetrols. Nordqvist et al. (*9*) obtained indications for the formation of tetrahydrotetrols in the metabolism of the *trans*-3,4-dihydrodiol of DBA and consequently proposed the dihydrodiol epoxide as an ultimate mutagenic metabolite of DBA (*9,25*). We, however, could not confirm the formation of tetrahydrotetrols from the M-region dihydrodiol of DBA (Table 1) but found evidence for more polar metabolites (*10*). We therefore proposed tetrol epoxides as ultimate mutagens of DBA (*10,11*).

Information on the structure of the ultimate mutagenic metabolite of the *trans*-5,6-dihydrodiol of DBA can be deducted from its microsomal metabolism (Table 1). Either the 5,6,10,11-bisdihydrodiol could give rise to the formation of a reactive tetrol epoxide (*10,11,25*) or the common precursor of the 5,6,10,11-bisdihydrodiol and the 5,6-dihydrodiol-11-phenol, the 5,6-dihydrodiol-10,11-epoxide, a nonvicinal dihydrodiol epoxide, could be the ultimate mutagen of the K-region dihydrodiol of DBA.

Conclusions

The present study has provided an example of how the regio- and/or stereoselectivity in the sequential attack of cytochrome P-450-dependent monooxygenase and microsomal epoxide hydrolase govern the genotoxicity of an environmentally relevant PAH. The contribution of the three *trans*-dihydrodiols formed by metabolic conversion at three distinct molecular regions of DBA, the bay, M, and K region, to its bacterial mutagenicity can be deduced from the results of investi-

gations concerning microsomal metabolism and mutagenic activity.

The bay-region (1,2-) dihydrodiol has probably no importance in the mutagenicity of DBA due to the fact that the *R,R* enantiomer, which is predominantly formed by liver microsomes of immature Sprague-Dawley rats after Aroclor 1254-pretreatment, exhibits only very weak bacterial mutagenicity.

The M-region (3,4-) dihydrodiol highly enriched in the *R,R* enantiomer is the principal metabolite of DBA. Since this enantiomer is activated to the most mutagenic species compared to all other enantiomers of the three *trans*-dihydrodiols, the M-region dihydrodiol of DBA very likely plays the most decisive role in the genotoxicity of this PAH.

The K-region (5,6-) dihydrodiol of DBA is metabolically activated to rather strong bacterial mutagens, a property unusual for K-region dihydrodiols of PAH. However, due to the preference of the cytochrome P-450 isozymes induced by Aroclor 1254 for metabolism at the M region of DBA, the *trans*-5,6-dihydrodiol is probably only of minor importance for the genotoxicity of DBA. This could change drastically if under enzymatic conditions different from those employed in this study the K region becomes the major site of attack by cytochrome P-450 dependent monooxygenases.

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